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# HUMAN MIXED LYMPHOCYTE CULTURES

## EVALUATION OF A MICROCULTURE TECHNIQUE UTILIZING THE MULTIPLE AUTOMATED SAMPLE HARVESTER (MASH)

G. B. THURMAN,* D. M. STRONG, A. AHMED, S. S. GREEN, K. W. SELL, R. J. HARTZMAN† AND F. H. BACH

*Experimental Immunology Division, Clinical Medical Sciences Department, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland* 20014, *and Departments of Medicine and Medical Genetics and Surgery, University of Wisconsin, Madison, Wisconsin* 53706, *U.S.A.*

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SUMMARY

Use of lymphocyte cultures for *in vitro* studies such as pretransplant histocompatibility testing has established the need for standardization of this technique. A microculture technique has been developed that has facilitated the culturing of lymphocytes and increased the quantity of cultures feasible, while lowering the variation between replicate samples. Cultures were prepared for determination of tritiated thymidine incorporation using a Multiple Automated Sample Harvester (MASH). Using this system, the parameters that influence the *in vitro* responsiveness of human lymphocytes to allogeneic lymphocytes have been investigated.

## INTRODUCTION

Interest in the *in vitro* reactivity of lymphocytes has greatly intensified since the discovery of their involvement in immunological function. Many clinical and research laboratories are now routinely doing short-term lymphocyte cultures. Examples of tests being run are: (1) determination of immunodeficiencies by testing the *in vitro* lymphocyte responsiveness of patients with diseases such as Hodgkin's disease (Jackson, Garret & Craig, 1970; Han & Sokal, 1970), chronic lymphocytic leukaemia (Han, 1971), thymic hypoplasia (Steele *et al.*, 1972), combined immunodeficiencies (Rádl *et al.*, 1971), subacute sclerosing panencephalitis (Lischner, Grover & DeForrest, 1971), etc.; (2) drug sensitivity testing (Halpern, Ky & Amache, 1967); (3) reactivity to tumour cells (Nagel & Holland, 1970); and (4) histocompatibility testing using mixed leucocyte cultures (Bain, Vas & Lowenstein, 1964; Bach & Hirschorn, 1964; Dausset, Ivanyi & Ivanyi, 1965; Albertini & Bach, 1968;

* Present address: Division of Biochemistry, University of Texas, Galveston Medical Branch, Galveston, Texas 77550, U.S.A.

† Present address: Mary Hitchcock Memorial Hospital, Hanover, New Hampshire 03755, U.S.A.

Correspondence: Dr Aftab Ahmed, Experimental Immunology Division, Clinical Medical Sciences Department, Naval Medical Research Institute, Bethesda, Maryland 20014, U.S.A.

Polet, 1972). This increased use of short-term lymphocyte cultures has pointed to the need for the development of new techniques and standardization of culture methods.

Bach, Solliday & Stambuk (1970), Bouroncle, Malspesis & Aschenbrand (1972), and Polet (1972) have described important parameters in mixed leucocyte cultures in culture tubes. We present here an evaluation of a method of doing mixed lymphocyte cultures in microtitre plates, and delineate important parameters influencing the *in vitro* response of lymphocytes. Also described is a rapid system of preparing cultures for scintillation spectrophotometry. This uses a Multiple Automated Sample Harvester (MASH) previously described (Hartzman *et al.*, 1972). The advantages of the microtitre plate for short-term cultures are readily recognized (Brody & Huntley, 1965; Hartzman *et al.*, 1971; Bach *et al.*, 1971; Parker & Lukes, 1971). The relatively small culture volume allows more tests per sample and permits testing of small children and leukopenic patients. Very little incubator space is required for a large number of cultures. Utilizing the MASH, one person can easily harvest ninety-six cultures, dry the filters and have them in the scintillation counter within 1 hr. This culture technique and automated harvesting procedure has resulted in a sharp decrease in the experimental error between replicate samples and a great saving in time.

## MATERIALS AND METHODS

The methods of obtaining and purifying lymphocytes have been adequately described elsewhere (Boyum, 1968; Thorsby & Brathe, 1970) but we will describe our method in brief. Venous, heparinized human blood was allowed to sediment 2–3 hr. The leucocyte-rich plasma was removed by expulsion through the bent needle of the syringe and centrifuged 10 min at 200 *g* to sediment the leucocytes. The platelet-rich plasma was removed and hard spun (2000 *g*, 20 min) to remove the platelets before it was used to supplement the culture media. The leucocytes were resuspended in culture media supplemented with antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml) and fresh L-glutamine (200 mM). This cell suspension was carefully layered on top of an equal volume of fresh filter-sterilized (0·2 μm Millipore) Ficoll–Hypaque gradient (sp. gr. 1·078) made by mixing four parts of an 8% (v/v) Ficoll (Sigma Chemical Co., St. Louis, Mo.) solution with one part of 50% Hypaque (Winthrop Laboratories, New York, N.Y.). The gradient and cell suspension was centrifuged for 40 min at ambient temperature providing 400 *g* at the interface. The band of cells at the media-gradient interface was carefully aspirated with a pipette and then washed once with media. This routinely gave 98–99% lymphocytes with about a 60% recovery. An aliquot of each donor's lymphocytes was removed and treated for 30 min with 25 μg/ml of mitomycin C (Calbiochem, La Jolla, Cal.) at 37°C to inhibit mitosis. They were washed twice and used as stimulating cells in the mixed lymphocyte cultures. Cells so treated are indicated in the data by the subscript 'm'.

The distribution of lymphocytes into the microtitre plates (Microtest II, Falcon Plastics, Oxnard, Calif. or Linbro IS series, Bellco Glass, Vineland, N.J.) was accomplished with Hamilton repeating dispensers (Hamilton Co., Whittier, Calif.). Since the cultures were 0·2 ml, three sizes of the Hamilton syringes were found most useful: 5·0 ml (delivers 0·10 ml per expulsion), 2·5 ml (delivers 0·05 ml) and 1·0 ml (delivers 0·02 ml). The syringes were fitted with $3\frac{1}{2}$ in 20G disposable spinal needles (Becton, Dickinson, Rutherford, N.J.) which facilitated handling of the cell suspensions and eliminated working directly over the

microtitre plate while dispensing the cells. Accuracy in dispensing the lymphocytes was essential for good reproducibility and small standard deviations within groups.

The cultures were incubated in a 5% $CO_2$ humidified atmosphere at 37°C. At the appropriate times before harvesting the cultures with the MASH, 0·02 ml of media containing methyl-$^3$H-thymidine ($^3$H-TdR, sp. act. 1·9 Ci/mM, Schwartz-Mann, Orangeburg, N.J.) was added. Essentially, the function of the MASH was to remove the contents of a row (twelve wells) of the plate, wash the wells, individually filter the fluids from each well and eliminate the unbound tritiated thymidine from the filter. Following the harvesting procedure, the filter strips were dried in a drying oven at 125°C for 15 min. The individual filter spot was then torn from the strip along the 'O' ring impression and placed in 1 dram Opticlear vials (Kimble, Toledo, Ohio). Three ml of scintillation cocktail made with toluene and Liquifluor (New England Nuclear, Boston, Mass.) was added, and the vials were capped and placed in lidless polyethylene scintillation vials (Packard Instrument Co., Downers Grove, Illinois). After cold and dark adaption of the vials for 10 min, the tritiated thymidine incorporation was determined by a Packard 2425 liquid scintillation spectrophotometer. The main advantage of this model counter was that it allowed loading and unloading of the scintillation vials in 50 vial trays rather than individually handling each vial as is necessary with endless belt models. The data from the scintillation counter were recorded on paper tape and the tape was used for data analysis with a Wang 700C Advanced Programming Calculator, which computed and printed the means and standard errors of replicate samples. This system provided fast and accurate analysis of the data and eliminated human error in manipulation of the raw data.

Our standard mixed lymphocyte culture technique consisted of 200,000 stimulating and 200,000 responding lymphocytes per culture with RPMI-1640 media ($NaHCO_3$ buffered) containing 10% plasma autologues to the responding cell. They were cultured in flat bottom plates for 5 days with a 1 $\mu$Ci tritiated thymidine pulse for the last 18 hr. The cultures were harvested onto glass fibre filters with only a saline wash utilizing the MASH. The data reported in this paper were obtained using this procedure unless otherwise indicated.

## RESULTS

It has been our general practice to use RPMI 1640 Media (GIBCO, Grand Island, N.Y.) buffered with sodium bicarbonate incubating the cultures in a 5% $CO_2$ atmosphere. However, Table 1 indicates that RPMI 1640 with sodium bicarbonate and 40 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, Cal. Biochem, La Jolla, Calif.) sealed with microtitre plate sealer (Cooke Engineering Co., Alexandria, Va.) gave higher tritiated thymidine incorporation than any other combination of media, buffers or atmospheres tested. Except for the HEPES buffered RPMI 1640 in a closed system, there seemed to be little consistent difference between RPMI 1640 and Medium 199 (Earle's Salts). As expected, the bicarbonate buffered cultures did poorly in a closed system.

The change in tritiated thymidine uptake with variation in cell concentration is shown in Table 2. An interpretation of the culture efficiency is made by expressing the gross counts per minute as cpm/50,000 total cells. This has shown that 200,000 stimulating and 200,000 responding lymphocytes per culture was close to the most efficient use of the cells. This represented a concentration of $1 \times 10^6$ responding and $1 \times 10^6$ stimulating lymphocytes per ml which is commonly used in the tube cultures.

TABLE 1. Human mixed lymphocyte cultures: media, buffers and atmosphere conditions

Average cpm (standard error) of the $^3$H-TdR incorporation in triplicate cultures

| Lymphocyte Mixture | RPMI 1640* | | | | Medium 199† (Earle's base) | | | |
|---|---|---|---|---|---|---|---|---|
| | Bicarbonate buffer | | Hepes buffer‡ | | Bicarbonate buffer | | Hepes buffer‡ | |
| | 5% $CO_2$ | Closed§ | 5% $CO_2$ | Closed | 5% $CO_2$ | Closed | 5% $CO_2$ | Closed |
| L × $L_m$¶ | 310 (14%) | 169 (7%) | 751 (22%) | 595 (10%) | 400 (19%) | 208 (17%) | 506 (7%) | 802 (8%) |
| M × $M_m$ | 205 (1%) | 174 (34%) | 289 (3%) | 250 (12%) | 268 (1%) | 157 (8%) | 152 (3%) | 171 (3%) |
| P × $P_m$ | 234 (15%) | 130 (13%) | 409 (24%) | 439 (6%) | 252 (32%) | 119 (7%) | 298 (20%) | 426 (24%) |
| L × $M_m$ | 27098 (5%) | 10923 (4%) | 27371 (2%) | 34899** (2%) | 29072 (3%) | 4601 (7%) | 23744 (5%) | 33390** (2%) |
| L × $P_m$ | 32742 (6%) | 16191 (4%) | 39304 (3%) | 50727** (6%) | 9028 (7%) | 1478 (19%) | 29820 (1%) | 35640 (4%) |
| M × $L_m$ | 31675 (4%) | 15317 (12%) | 37838** (6%) | 45171** (3%) | 27099 (4%) | 10919 (4%) | 26696 (5%) | 28674 (5%) |
| M × $P_m$ | 27519 (2%) | 5068 (6%) | 34510 (6%) | 41383** (6%) | — | — | 14345 (14%) | 18698 (8%) |
| P × $L_m$ | 28481 (4%) | 6984 (9%) | 27085** (9%) | 35123** (9%) | 31211** (5%) | 7630 (19%) | 25791 (7%) | 33552** (4%) |
| P × $M_m$ | 15058 (9%) | 1053 (16%) | 19669 (1%) | 26624** (1%) | 11695 (16%) | 930 (28%) | 16268 (6%) | 22112 (3%) |
| $L_m$ × $M_m$ | 86 (15%) | 58 (11%) | 110 (19%) | 88 (25%) | 184 (19%) | — | 161 (13%) | — |
| $L_m$ × $M_m$ | 115 (16%) | 60 (5%) | 162 (3%) | 211 (40%) | 742 (25%) | — | 288 (20%) | — |
| $M_m$ × $P_m$ | 84 (20%) | 54 (3%) | 92 (7%) | 72 (4%) | 61 (18%) | — | 131 (20%) | — |

* 2000 mg $NaHCO_3$/litre.
† 2200 mg $NaHCO_3$/litre.
‡ 40 mM.
§ Sealed air tight with sealer tape.
¶ Subscript 'm' indicates mitomycin C treatment. Lymphocytes from three individuals (L, M and P) were used.
** Statistically the highest for that lymphocyte mixture.

Fig. 1 shows that in our system, the variation between use of autologous and normal homologous plasma was negligible and that there was little difference as long as 4% or more plasma was used. The peak of the curves in Fig. 1 is broad, but reaches its maximum

TABLE 2. Human mixed lymphocyte culture: cell concentrations

| Average cpm (standard error of the $^3$H-TdR incorporation in triplicate cultures) | | | | |
|---|---|---|---|---|
| Stimulating lymphocytes* per well | Responding lymphocytes (R) per well | | | |
| | 50,000 | 100,000 | 200,000 | 400,000 |
| Gross counts | | | | |
| $R_m$ (controls) | | | | |
| 50,000 | 81 (23%) | 66 (9%) | 75 (11%) | 70 (3%) |
| 100,000 | 300 (35%) | 150 (38%) | 162 (38%) | 161 (35%) |
| 200,000 | 675 (15%) | 265 (54%) | 270 (39%) | 295 (33%) |
| 400,000 | 731 (4%) | 401 (44%) | 325 (39%) | 423 (43%) |
| $S_m$ | | | | |
| 50,000 | 138 (27%) | 776 (31%) | 5031 (25%) | 11610 (8%) |
| 100,000 | 1392 (17%) | 5359 (10%) | 16455 (11%) | 20722 (2%) |
| 200,000 | 4158 (15%) | 12248 (9%) | 25256 (4%) | 28968 (3%) |
| 400,000 | 6909 (12%) | 17504 (6%) | 31275 (4%) | 35296 (1%) |
| $T_m$ | | | | |
| 50,000 | 614 (15%) | 1921 (24%) | 5031 (12%) | 10312 (3%) |
| 100,000 | 3447 (30%) | 9341 (18%) | 15461 (4%) | 16238 (5%) |
| 200,000 | 5776 (24%) | 14744 (14%) | 25652 (10%) | 30753 (9%) |
| 400,000 | 12149 (10%) | 23978 (8%) | 35687 (5%) | 37427 (1%) |
| Counts per 50,000 total cells | | | | |
| $R_m$ | | | | |
| 50,000 | 40 | 22 | 15 | 8 |
| 100,000 | 100 | 38 | 27 | 15 |
| 200,000 | 135 | 44 | 34 | 25 |
| 400,000 | 81 | 40 | 27 | 26 |
| $S_m$ | | | | |
| 50,000 | 69 | 259 | 1006 | 1290 |
| 100,000 | 464 | 1340 | 2742 | 2072 |
| 200,000 | 832 | 2041 | 3157 | 2414 |
| 400,000 | 768 | 1750 | 2606 | 2606 |
| $T_m$ | | | | |
| 50,000 | 307 | 640 | 1006 | 1146 |
| 100,000 | 1149 | 2335 | 2577 | 1476 |
| 200,000 | 1155 | 2457 | 3206 | 2563 |
| 400,000 | 1350 | 2398 | 2974 | 2399 |

* Subscript 'm' indicates mitomycin C treatment. Lymphocytes from three individuals (R, S and T) were used.

around 10–12%. The incubation time for maximum response as shown in Fig. 2 varied between 5–6 days in the microtitre plate. However, stimulation was evident as early as 2 days.

Several experiments were run comparing our 'tube' method of mixed lymphocyte cultures (Green & Sell, 1970) with our microtitre plate method and a representative experiment is presented in Table 3. The round bottom plates gave results equivalent to those obtained with

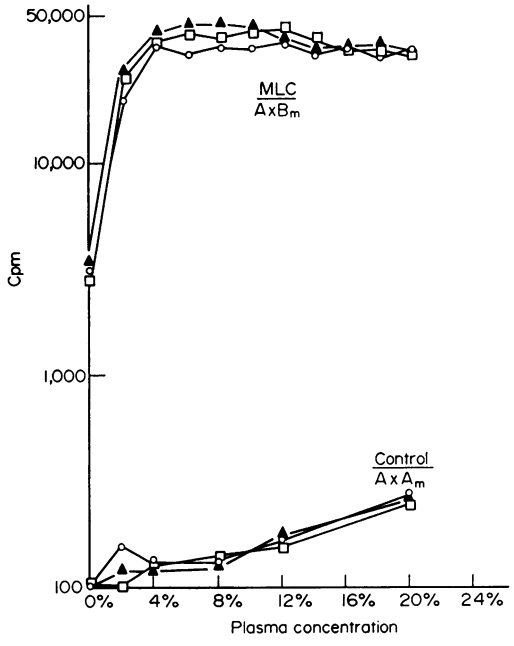


FIG. 1. Effect of plasma source and concentration on human one-way mix lymphocyte microcultures. Homologous plasmas 1 and 2 are fresh nonpooled plasmas from two separate donors. Responding cells are from individual A and stimulating cells are from individual B. Mitomycin-C treatment is indicated by the subscript 'm'. (○) Autologous. (□) Homologous 1. (▲) Homologous 2.

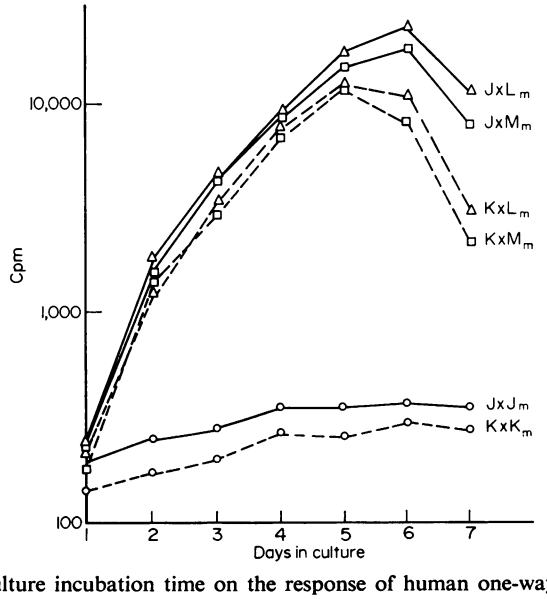


FIG. 2. Effect of culture incubation time on the response of human one-way mixed lymphocyte microcultures. Responding cells are from two individuals (J and K) and stimulating cells are from two different individuals (L and M). Mitomycin C treatment is indicated by the subscript 'm'. (—) J lymphocytes. (- - -) K lymphocytes. (○) Control. (△) $L_m$ lymphocytes. (□) $M_m$ lymphocytes.

the culture tube method, whereas flat bottom plates gave lower tritiated thymidine uptake. Another experiment (Table 4) also showed that both round and V bottom plates usually gave slightly higher gross counts in the mixed lymphocyte cultures than did the flat bottom plates. However, the controls in the round and V bottom plates were always higher than identical controls in the flat bottom plates.

TABLE 3. Human mixed lymphocyte cultures. Culture tubes versus microtitre plates

| | Average cpm of H-TdR incorporation in triplicate cultures | | |
|---|---|---|---|
| Lymphocyte mixture* | Culture tubes† | Flat bottom microtitre plates‡ | Round bottom microtitre plates‡ |
| Two way | | | |
| X×X | 4217 | 1224 | 3797 |
| Y×Y | 1374 | 1005 | 1597 |
| Z×Z | 2006 | 1111 | 1920 |
| X×Y | 21359 | 20088 | 22517 |
| X×Z | 21830 | 19117 | 29086 |
| Y×Z | 16565 | 19844 | 26825 |
| Mitomycin C blocked (25 μg/ml, 30 min, 37°C) | | | |
| $X \times X_m$ | 2912 | 388 | 1330 |
| $Y \times Y_m$ | 1729 | 654 | 1381 |
| $Z \times Z_m$ | 1538 | 507 | 1741 |
| $X \times Y_m$ | 14360 | 8921 | 13587 |
| $X \times Z_m$ | 14911 | 10879 | 16373 |
| $Y \times X_m$ | 10208 | 5516 | 12474 |
| $Y \times Z_m$ | 9630 | 9640 | 13396 |
| $Z \times X_m$ | 17546 | 6657 | 20078 |
| $Z \times Y_m$ | 17370 | 18963 | 21488 |

* Lymphocytes from three individuals (X, Y and Z) were used.

† Standard disposable 16×100 glass culture tubes were used (Green & Sell, 1970). The cpm given under culture tubes is 20% of the gross count, since five times as many cells were used in macro as in micro. Standard errors average about 16%.

‡ Standard errors average about 8%.

*Labelling and harvesting*

Varying amounts of tritiated thymidine were added to mixed lymphocyte cultures 18 hr before harvesting and the results are shown in Fig. 4. The trend was for the cultures to reach maximum thymidine incorporation around 1 μCi per culture. This represents adding 0·13 μg of thymidine per well (0·2 ml). A pulse-duration experiment showed that an 8-hr pulse gave optimum counts (Fig. 5). An 18-hr overnight pulse had been used as a matter of convenience but now an 8-hr pulse is utilized.

A variety of filter types can be used with the MASH. Table 5 lists five types used in one experiment. The Reeve Angel 934AH glass fibre filter was the most economical and had the least resistance to fluid and air flow. To obtain enough draw with Millipore filters, the pore size had to be 5 μm or greater. The 5 μm Millipore filters gave results quite compatible with those given by the glass fibre filters. However, the 8 μm filters showed loss of over 50% of

TABLE 4. Human mixed lymphocyte cultures: microculture plate type

| Lymphocyte mixture | Average cpm (standard error) of the $^3$H-TdR incorporation in quadruplicate cultures | | |
|---|---|---|---|
| | Plate type | | |
| | Flat bottom | Round bottom | V Bottom |
| $K \times K_m$* | 206 (7%) | 390 (28%) | 471† (8%) |
| $L \times L_m$ | 342 (22%) | 898 (25%) | 718† (3%) |
| $M \times M_m$ | 317 (25%) | 643 (24%) | 892† (20%) |
| $K_m \times L_m$ | 965 (3%) | 2032† (4%) | 1594† (4%) |
| $L_m \times M_m$ | 1436 (10%) | 3474† (7%) | 3270† (7%) |
| $M_m \times K_m$ | 1287 (8%) | 3137† (5%) | 2699† (6%) |
| $K \times L_m$ | 15620 (2%) | 19195† (5%) | 20068† (6%) |
| $K \times M_m$ | 15450 (2%) | 19786† (6%) | 20002† (5%) |
| $L \times K_m$ | 27467 (4%) | 29112† (3%) | 32629† (1%) |
| $L \times M_m$ | 32047 (1%) | 30604 (5%) | 28979 (4%) |
| $M \times K_m$ | 23128 (8%) | 26838 (2%) | 28066 (6%) |
| $M \times L_m$ | 22915 (4%) | 25639 (3%) | 26625† (1%) |

* Subscript 'm' indicates mitomycin C treatment. Lymphocytes from three individuals (K, L and M) were used.
† Significantly higher than flat bottom plate values.

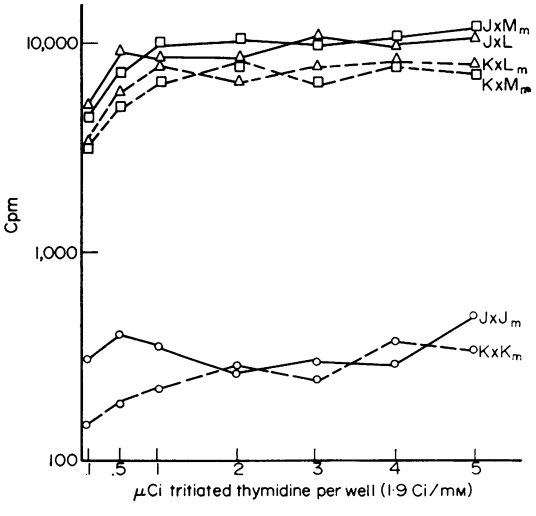


FIG. 3. Assessment of the amount of tritiated thymidine (sp. act. 1·9 Ci/mM) necessary for maximum incorporation of the label in human one-way mixed lymphocyte microcultures. Responding cells are from two individuals (J and K) and stimulating cells are from two different individuals (L and M). Mitomycin-C treatment is indiciated by subscript 'm'. (—) J lymphocytes. (- - -) K lymphocytes. (○) Control. (△) $L_m$ lymphocytes. (□) $M_m$ lymphocytes.

the activity implying that some of the bound label passed through an 8 $\mu$m pore but could not pass through a 5 $\mu$m pore. Table 5 also indicates that after incubation and labelling, the cultures can be stored at 4°C in a regular atmosphere for at least a week before harvesting. Little effect on the amount of tritiated thymidine incorporated is observed when harvesting is delayed in this manner.

The general procedure for preparing lymphocyte cultures for $^3$H-TdR uptake determination has been to precipitate the DNA with trichloroacetic acid followed by a methanol wash to remove water that quenches scintillation. In our system essentially the same results were obtained washing with normal saline alone, as shown in Table 6. Most of the bound activity was deposited on the filter in the first 3 ml of wash fluid, and the remaining fluid

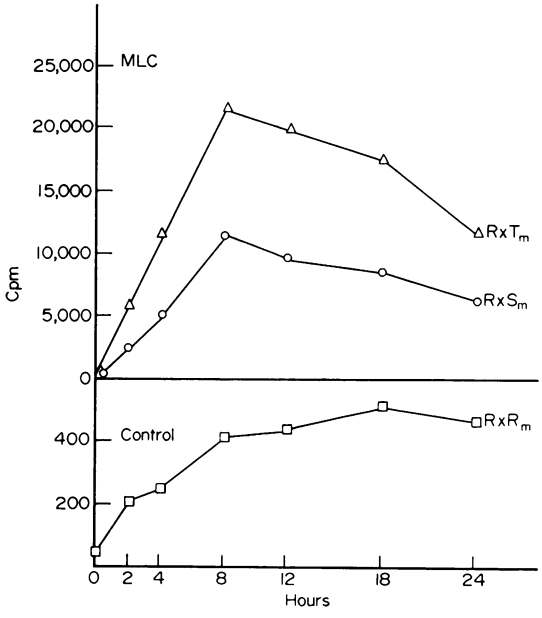


FIG. 4. Tritiated thymidine pulse duration curve for human one-way mixed lymphocyte microcultures. Responding cells are from one individual (R) and stimulation cells are from two individuals (S and T). Mitomycin-C treatment is indicated by a subscript 'm'.

served just to wash the unbound label through the filter. There was no significant cross contamination from well to well nor was there any carry over from one row of wells to the next. A check for retention of unbound radioisotope on the filter in the MASH was done by harvesting 5 $\mu$Ci of tritiated thymidine per well in media as lymphocyte cultures were harvested. The results (background controls, Table 5) showed little, if any, retention of unbound label.

*Determination of radioisotope Incorporation*

The 1 dram vials used as scintillation vials gave a counting efficiency of about 39% (unbound tritiated thymidine in 3 ml of scintillation fluid) while the efficiency was 42% with regular polyethylene vials (with 15 ml of scintillation fluid). The bound activity from

TABLE 5. Human mixed lymphocyte cultures: filter types and processing technique

| | Average cpm (standard error) of the $^3$H-TdR incorporation in sextuplicate cultures | | | | | | |
|---|---|---|---|---|---|---|---|
| | Glass fibre filters* | Millipore† | | Nucleopore‡ | Scintillation pads | Glass fibre filters | |
| | | 5 μm | 8 μm | 5 μm | | | |
| Unbound retention test | | | | | | 4 days | 7 days |
| Background | 38 (4%) | 38 (4%) | 38 (4%) | 38 (4%) | 38 (4%) | storage | storage |
| Media + 5 μCi $^3$H-Tdr | 40 (4%) | 74 (8%) | | 43 (6%) | 43 (2%) | at 4°C | at 4°C |
| Mixed lymphocyte cultures** | | | | | | | |
| Regular processings | | | | | | | |
| P control | 384 (13%) | 268 (12%) | 182 (38%) | 581 (17%) | 204 (9%) | 457 (13%) | 1208 (19%) |
| T control | 469 (21%) | 421 (11%) | 128 (29%) | 405 (16%) | 221 (17%) | 663 (19%) | 720 (25%) |
| $P \times S_m$ | 14352 (3%) | 12266 (5%) | 5575 (20%) | 10634 (5%) | 10682 (5%) | 16380 (3%) | 16627 (5%) |
| $T \times S_m$ | 21695 (6%) | 21562 (4%) | 8330 (16%) | 16715 (7%) | 18138 (6%) | 24075 (5%) | 26558 (2%) |
| Sample oxidizer | | | | | | | |
| P control | †† | 300 (12%) | 190 (21%) | 374 (23%) | 308 (20%) | | |
| T control | †† | 466 (19%) | 812 (16%) | 685 (26%) | 408 (25%) | | |
| $P \times S_m$ | †† | 11893 (15%) | 4734 (24%) | 20937 (11%) | 15027 (12%) | | |
| $T \times S_m$ | †† | 19581 (21%) | 12268 (14%) | 22333 (9%) | 30649 (12%) | | |

* Reeve Angel glass fiber filter 934AH.
† MF, Millipore mixed esters of cellulose filters SM and SC.
‡ General Electric Nucleopore membrane filters.
§ Whatman 3 MM qualitative filter papers EDP No. 4752–N10 Scintillation Pads.
¶ Harvested as regular cultures, processed the regular way.
** Subscript 'm' indicates mitomycin C treatment. Lymphocytes from three individuals (P, S and T) were used.
†† Glass fibre filters cannot be processed in the sample oxidizer.

harvested cultures was found to remain on the glass fibre filter spots and in this case the 1 dram vials were as efficient as the regular scintillation vials while being more economical to use. It was found that the dried filters had very little quenching effect in the scintillation fluid. The standard deviation of the quench values (automatic external standard and channels ratio) of many samples was less than 1%. With little variation in quenching, the problem of conversion to disintegrations per minute is avoided since it does not alter the comparative results obtained.

The use of a sample oxidizer (Packard Instrument Co., Downer's Grove, Ill.) should give a higher recovery of measurable label. Although preliminary work indicated this, a detailed experiment using different filter types, Table 5, showed that the values obtained using the sample oxidizer had a higher standard error and did not always give higher recovery. Unfortunately, the glass fibre filters could not be used in the sample oxidizer since glass fibre will not burn but melts. The technique using the sample oxidizer is tedious (about 2 min per sample) and the advantages too few to warrant recommending its use in regular assays such as ours.

TABLE 6. Human mixed lymphocytes cultures: washing procedures in harvesting cultures

| | Average cpm (standard error) of $^3$H-TdR incorporation in triplicate cultures | | | |
|---|---|---|---|---|
| Processing* | $P \times P_m$† | $T \times T_m$ | $P \times X_m$ | $T \times X_m$ |
| Saline | 384 (13%) | 469 (21%) | 14353 (3%) | 21695 (6%) |
| Saline-TCA | 361 (18%) | 1514 (18%) | 13737 (3%) | 21051 (6%) |
| Saline-TCA-methanol | 541 (25%) | 752 (20%) | 14439 (3%) | 20366 (8%) |
| Saline | 227 (14%) | 859 (21%) | 13240 (5%) | 23566 (8%) |

* Normal saline (9 g/litre) and 5% TCA was used. Processing involved harvesting each culture and washing each microtitre well with at least 5 ml of the indicating fluid.

† Subscript 'm' indicates mitomycin C treatment. Lymphocytes from three individuals (P, T and X) were used.

## DISCUSSION

The optimum conditions for human mixed lymphocyte cultures are important for obtaining statistically reliable results. The inherent variation in the natural response makes it advantageous to eliminate as many variations in the culture condition as possible. The rapid rise in the pH of bicarbonate buffered medias during culture preparation is a problem that can be eliminated by the use of HEPES buffer. The marked effect of pH variations in bicarbonate buffered media on the growth and metabolism of cultured mammalian cells has been noted by Eagle (1971). HEPES buffer will not maintain a constant pH over long periods of time but rather tends to stabilize the pH of the media by resisting rapid changes. Darzynkiewicz & Jacobson (1971) compared bicarbonate and HEPES buffers in lymphocyte cultures and obtained almost identical results, except at higher cell concentrations, where HEPES gave greater incorporation of tritiated thymidine. They found that the optimum response to PHA-P was at a pH range of 7·4–7·6. The bicarbonate buffered medias we used started at a pH of 7·6 but rapidly went to 8·2 when exposed to regular atmosphere.

The HEPES buffered media remained at 7·6. Eagle recommends a HEPES concentration of 10–15 mM (in combination with other buffers). Medium 199 and RPMI 1640 containing 25 mM HEPES with compensation for sodium osmolarity are commercially available (GIBCO, Grand Island, New York). HEPES concentrations higher than 50 mM have proved to be somewhat toxic to cells and increased the tonicity of the media beyond the tolerable range of some cell types.

The optimum cell concentration in the micro system varies depending upon the cell source. We have found that 200,000 stimulating and 200,000 responding lymphocytes per culture consistently gave good results. Higher gross counts can be obtained using more cells but the amount of stimulation per cell number generally decreases after this concentration thus lowering the culture efficiency.

The plasma types and concentrations are variables which can greatly influence the interpretation of the results. The discovery of blocking or inhibitory factors in the plasma of certain patients complicates the picture. Culturing in autologous plasma is the most representative of the related *in vivo* response; however, it is difficult to make *in vitro* comparisons unless various plasma and cell type combinations are also cultured. The importance of the innovation of lymphocyte microcultures and the Multiple Automated Sample Harvester becomes obvious when such multiple combinations are set up and harvested.

Round and V bottom plates generally gave higher tritiated thymidine incorporation in human mixed lymphocyte cultures than did flat bottom plates; however, flat bottom plates offer the advantage of a larger working capacity (0·4 ml versus 0·25 ml). The tritiated thymidine incorporation obtained in any of the three plate types was sufficiently high to give good results. The higher control values always seen in round and V bottom plates are probably related to the fact that the cells settle out into a button in these plates rather than a thin sheet. This sheds some doubt on the use of stimulation indices to express experimental data. Such indices place undue emphasis on control values which can widely vary within a single experiment. The standard error on replicate control cultures in our system was often over 20%, while stimulated cultures' standard error averaged under 10%. Stimulation indices are useful for some comparisons but should be used in conjunction with the original data to give others a complete perspective of the experimental results.

The kinetics of thymidine incorporation into human mixed lymphocyte cultures have been well studied. Bain (1970) chose 0·5 μCi/ml of 0·48 Ci/mM tritiated thymidine (0·25 μg/ml) as an optimum for a 4-hr pulse. Her data suggests that the rate of tritiated thymidine incorporation is approximately linear up to 6 hr (for sp. act. greater than 1·0 Ci/mM and a 1 μCi/ml dose) and that maximum incorporation occurs at eight hours after which the incorporation decreases. Our pulse duration curve (Fig. 4) shows linearily up to 8 hr with a significant reduction thereafter. Caspary and Hughes (1972) determined that 0·5 μCi/ml of 5 Ci/mM tritiated thymidine (0·027 μg/ml) was optimum for mixed lymphocyte cultures. However, their data indicated that the optimum dose for maximum tritiated thymidine incorporation is greater than their largest dose of 2·27 μCi/ml. Fig. 3 shows our optimum of 1 μCi/well (5 μCi/ml at which point additional tritiated thymidine (up to 25 μCi/ml) had no effect on the total amount incorporated. This suggests that a point of flooding the system with thymidine had been reached, but the point of chemical or radiation toxicity has not yet been reached. Sample and Chretien (1971) have shown that for PHA-stimulated human leucocytes 24·2 μg/ml of thymidine had to be added to achieve flooding conditions.

Lower amounts of tritiated thymidine are incorporated in mixed lymphocyte cultures than in PHA-stimulated cultures so flooding conditions should be reached with a lower amount of thymidine. Our optimum of 1 $\mu$Ci/well (5 $\mu$Ci/ml) is equivalent to adding 0·6375 $\mu$g thymidine/ml. Shons *et al.* (1972) investigated the effect of specific activity of tritiated thymidine and length of incubation on mixed leucocyte microcultures and concluded that the optimum was the addition of 2 $\mu$Ci of 17·3 Ci/mM tritiated thymidine per 200,000 responding lymphocytes for an exposure of 18–24 hours. It is fortunate that for comparative purposes the amount of tritiated thymidine added is not a very critical parameter as long as it remains constant within a single experiment.

The values of a microculture system for lymphocyte cultures are many fold and obvious, compared to a system using culture tubes. The major advantages are the savings in time and materials thus allowing more cultures to be evaluated per sample in a given amount of time with increased accuracy. In our laboratories the increase in experimental capability provided by the microculture system and the MASH has been dramatic.

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